Oxidative stress during peritoneal dialysis: Implications in functional and structural changes in the membrane

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Progressive peritoneal fibrosis, membrane hyperpermeability, and ultrafiltration failure have been observed in patients on long-term peritoneal dialysis (PD). The present study tested the hypothesis that reactive oxygen species (ROS) generated by conventional PD solution (PDS) mediate functional and structural alterations of peritoneal membrane *in vivo*. Sprague–Dawley rats were randomized to control, PDS, PDS with an antioxidant, and PDS with an angiotensin II (Ang II) receptor blocker. Commercial PDS containing 3.86% glucose (20–30 ml) with or without *N*-acetylcystein (NAC) 10 mM or losartan 5 mg/kg was administered intraperitoneally twice a day for 12 weeks. Control rats received sham injection. Rats treated with PDS had significantly lower drain volume and D_4/D_0 glucose, but higher D_4/P_4 creatinine and increased membrane thickness and endothelial NOS (eNOS) expression compared to control rats. Omental transforming growth factor (TGF)-β1, vascular endothelial growth factor (VEGF), collagen I, and heat-shock protein (hsp) 47 expression and lipid peroxide levels and dialysate VEGF and Ang II concentrations were significantly increased in rats treated with PDS compared to control. All of these changes were prevented by both NAC and losartan. In conclusion, the present study demonstrates that ROS generated by conventional PDS are, in large part, responsible for peritoneal fibrosis and membrane hyperpermeability. We suggest that antioxidants or Ang II receptor blockers may allow better preservation of the structural and functional integrity of the peritoneal membrane during long-term PD.

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Long-term peritoneal dialysis (PD) is associated with progressive increase in the thickness of peritoneal membrane, predominantly in the submesothelial compact collagenous zone,[1] and membrane hyperpermeability.[2] The mechanisms involved in these structural and functional changes remain unclear, but prolonged exposure of the membrane to conventional PD solution (PDS) containing high concentrations of glucose and glucose degradation products (GDP) may play an important role. We have previously demonstrated that conventional high glucose-containing PDS increases transforming growth factor-β1 (TGF-β1), vascular endothelial growth factor (VEGF), and procollagen III N-terminal peptide secretion by human peritoneal mesothelial cells (HPMC),[3] and that high glucose-induced activation of protein kinase C mediates TGF-β1 and fibronectin upregulation in HPMC.[4] We also showed that conventional PDS[5] and high glucose[6] increase cellular reactive oxygen species (ROS) in HPMC, that high glucose-induced ROS generation is dependent on activation of protein kinase C, nicotinamide adenine dinucleotide phosphate oxidase, and mitochondrial metabolism, and that ROS provide signal amplification in high glucose-induced fibronectin expression by HPMC.[6] Recently, we demonstrated that high glucose increases cellular and secreted angiotensin II (Ang II) in HPMC, that Ang II induces TGF-β1 and fibronectin upregulation through generation of cellular ROS, and that losartan and captopril significantly reduce high glucose-induced TGF-β1 and fibronectin secretion by HPMC.[7]

Mortier *et al.*[8,9] demonstrated that chronic exposure of rat peritoneum to conventional PDS containing high concentrations of glucose and GDP resulted in loss of ultrafiltration capacity, increased vascular density, development of fibrosis, and higher accumulation of advanced glycation end-products but not when exposed to new solution containing low concentration of GDP, suggesting that GDP may play a role in the PDS-induced peritoneal changes. GDP can generate intracellular ROS and signal through ROS. Methylglyoxal-induced heparin-binding epidermal growth factor-like growth factor mRNA expression in rat aortic smooth muscle cells[10] and methylglyoxal-induced VEGF secretion by HPMC (HB Lee *et al.*, unpublished data) were shown to be suppres-

sed by antioxidant *N*-acetylcystein (NAC). Acetaldehyde-induced α1(I) procollagen gene expression in mouse hepatic stellate cells was also blocked by catalase.[11] GDP are precursors of advanced glycation end-products, which also induce cellular ROS by direct action[12] and through binding their receptor for advanced glycation end-products[13] and signal through ROS.[14] ROS, in turn, promote advanced glycation end-products formation[15] and can thereby amplify advanced glycation end-products signal.

In this study, we tested our hypothesis that ROS generated by conventional PDS, regardless of whether they are from high glucose, GDP, or Ang II, may play a role in peritoneal membrane alterations *in vivo* and that antioxidant may prevent such changes. We specifically examined the effect of Ang II type 1 receptor blocker in PDS-induced membrane alterations in view of the fact that Ang II generates and signals through ROS in HPMC.[7]

RESULTS

Peritoneal permeability and drained volume

At the end of 12 weeks, rats treated with PDS had significantly lower drain volume and D_4/D_0 glucose, but higher D_4/P_4 creatinine compared to control rats. Both NAC and losartan prevented the decrease in drain volume and D_4/D_0 glucose and the increase in D_4/P_4 creatinine (Figure 1).

Peritoneal membrane thickness

Subhepatic peritoneal membrane showed intact mesothelial cell monolayer in control rats (Figure 2a). In rats treated with PDS, a significant increase in peritoneal thickness with collagen deposition in submesothelial layer was observed (Figure 2a). Both NAC and losartan prevented the increase in thickness of both visceral and parietal peritoneum (Figure 2a and b).

Expression of eNOS

In control rats, a faint signal for endothelial NOS (eNOS) was located in the endothelial lining. In rats treated with PDS, there was an intense eNOS signal along the vessel wall. Both NAC and losartan attenuated the increase in the signal (Figure 3).

Omental collagen I and hsp 47 expression

Omental collagen I and heat-shock protein (hsp) 47 expression was significantly increased in rats treated with PDS compared to control rats. Both NAC and losartan prevented the increase (Figure 4).

Omental TGF-β1, VEGF, and LPO

Omental TGF-β1 (Figure 5a) and VEGF (Figure 5b) expression was significantly increased in rats treated with PDS compared to control. Both NAC and losartan significantly inhibited PDS-induced increase in TGF-β1 and VEGF. Omental lipid peroxide (LPO) (Figure 5c) also increased in rats treated with PDS, although the difference did not reach statistical significance. Losartan significantly reduced LPO in omentum when compared to rats treated with PDS without NAC or losartan.

Dialysate TGF-β1, VEGF, and Ang II

Dialysate TGF-β1 also increased in rats treated with PDS and the treatment with both NAC and losartan attenuated the increase, although the changes did not reach statistical significance (Figure 6a). Dialysate VEGF (Figure 6b) and Ang II (Figure 6c) concentrations significantly increased in rats treated with PDS compared to control and both NAC and losartan significantly inhibited PDS-induced increase in VEGF and Ang II.

DISCUSSION

In this study, we hypothesized that cellular ROS generated by conventional PDS may play a role in structural and functional peritoneal changes observed during long-term PD and that antioxidant may prevent such changes.

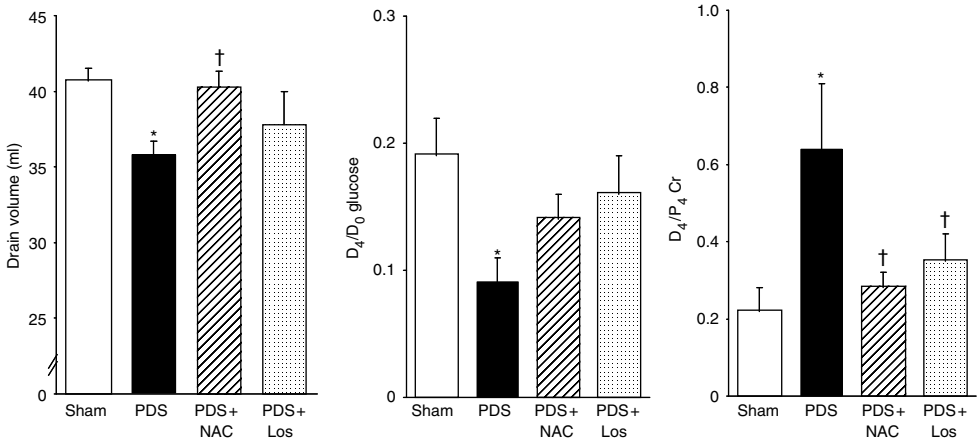

Figure 1 | Effect of PDS with or without NAC or losartan on peritoneal permeability and ultrafiltration capacity. Peritoneal equilibration test using 4.25% glucose PDS was performed immediately before being killed. Values are mean ± s.e. of seven to eight animals. *$P < 0.05$ versus control; †$P < 0.05$ versus PDS-treated rats.

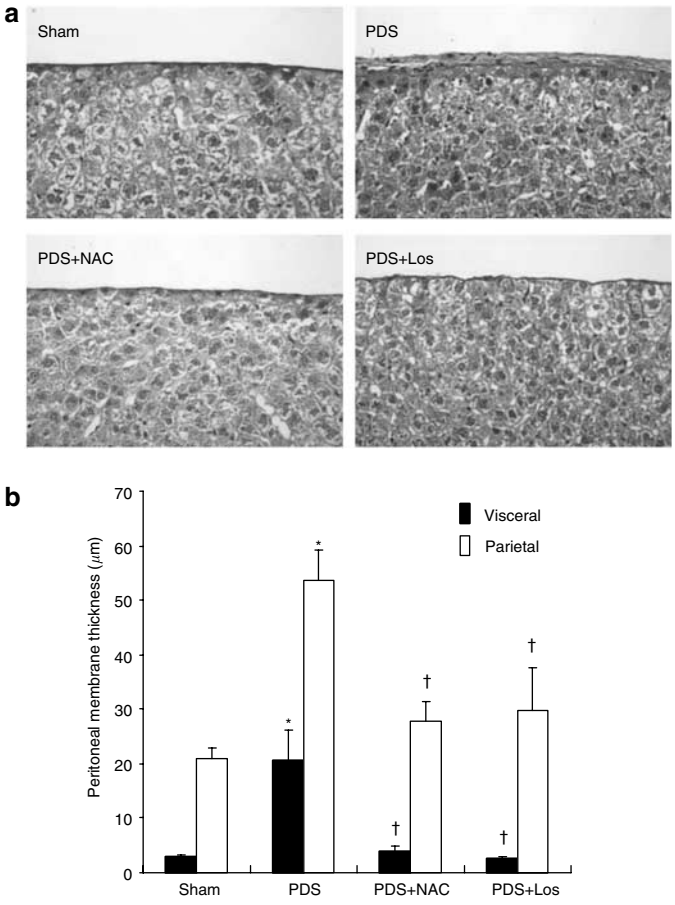

Figure 2 | Effect of NAC or losartan on PDS-induced peritoneal membrane thickening. (**a**) Masson's trichrome staining of subhepatic peritoneal membrane was performed as described in Materials and Methods. Original magnification, × 400. (**b**) Maximal peritoneal membrane thickness was measured by Image Pro Plus as described in Materials and Methods. Values are mean ± s.e. of seven to eight animals. *$P < 0.05$ versus control; †$P < 0.05$ versus PDS-treated rats.

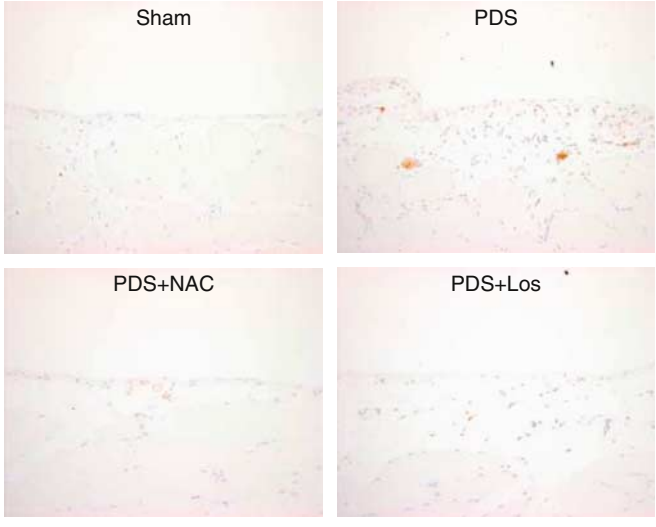

Figure 3 | Effect of NAC and losartan on PDS-induced expression of eNOS. Immunohistochemical staining for eNOS in the parietal peritoneal membrane was performed as described in Materials and Methods. Original magnification, × 400.

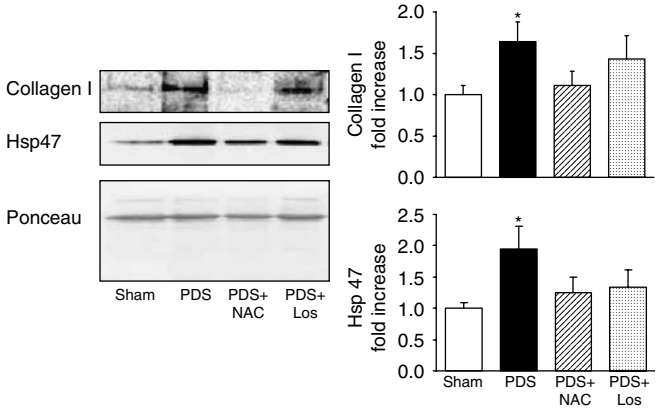

Figure 4 | Effect of NAC or losartan on PDS-induced expression of collagen I and hsp 47. Protein was extracted from omental tissue and was subjected to Western blot analysis. Left panel shows a representative Western blot, and right panel represents relative increase as mean ± s.e. of seven to eight animals. Equal amount of samples were verified by Ponceau S staining. *$P < 0.05$ versus control.

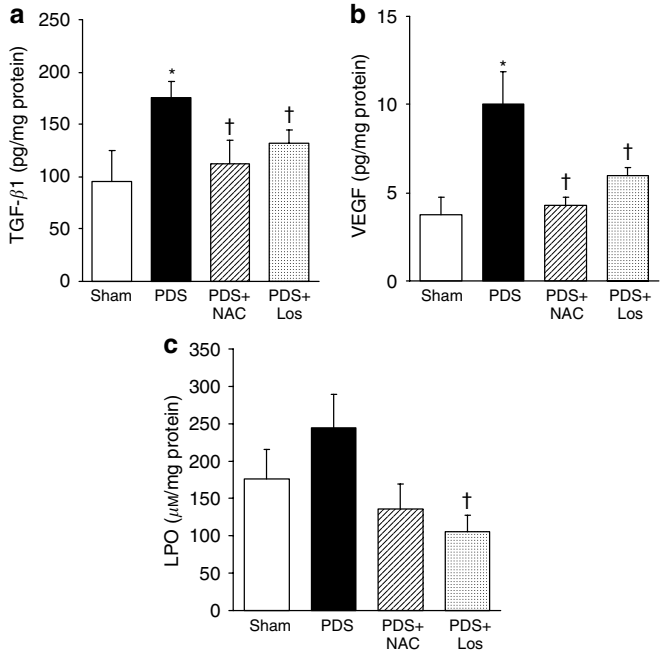

Figure 5 | Effect of NAC or losartan on (a) PDS-induced omental TGF-β1, (b) VEGF, and (c) LPO levels. Omental TGF-β1 and VEGF content was measured by ELISA and LPO by modified thiobarbituric acid method as described in Materials and Methods. Values are mean ± s.e. of six to eight animals. *$P<0.05$ versus control; †$P<0.05$ versus PDS-treated rats.

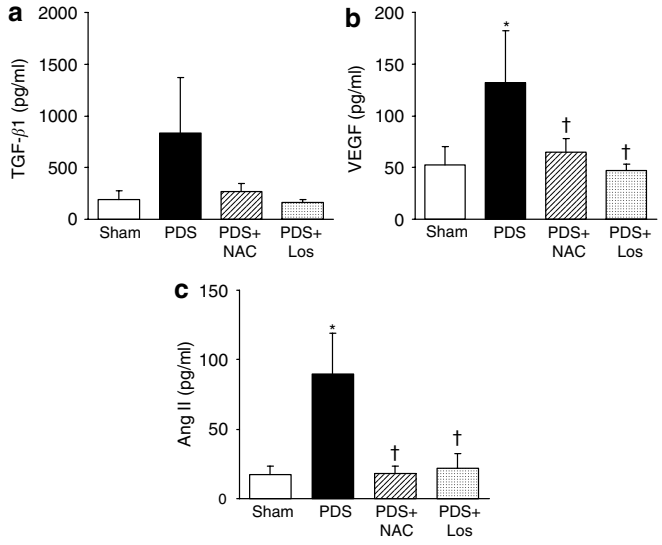

Figure 6 | Effect of PDS with or without NAC or losartan on (a) dialysis effluent concentrations of TGF-β1, (b) VEGF, and (c) Ang II. TGF-β1, VEGF, and Ang II concentrations were measured by ELISA as described in Materials and Methods. Values are mean ± s.e. of five to eight animals. *$P<0.05$ versus control; †$P<0.05$ versus PDS-treated rats.

In our animal model of PD where the peritoneum was exposed to PDS over 12 weeks, we observed changes similar to those observed in long-term PD patients with increased transport of creatinine and glucose, decreased ultrafiltration capacity, and increased peritoneal thickening and new vessel formation. In rats treated with PDS, thickening of peritoneal membrane was observed in both parietal and visceral peritoneum and this was associated with increased omental collagen I and hsp 47 expression. Hsp 47 is a collagen-specific molecular chaperon during the synthesis and/or secretion of procollagen.[16] In various renal diseases including renal interstitial fibrosis induced by unilateral ureteral obstruction,[17] experimental mesangioproliferative glomerulonephritis,[18] and age-related nephropathy,[19] the expression of hsp 47 was shown to be correlated with the degree of collagen expression. Recently, Mishima *et al.*[20] showed marked increase in hsp 47 expression in mesothelial cells and in submesothelial connective tissue in chlorhexidine gluconate-induced peritoneal fibrosis. These observations together with our finding in this study suggest that hsp 47 is a good marker of collagen accumulation in the kidney and peritoneum. Our finding that antioxidant NAC prevented PDS-induced collagen I and hsp 47 accumulation in the omentum strongly suggests that ROS are the major mediator of peritoneal fibrosis.

TGF-β1 is a multifunctional cytokine that is central in the process of fibrogenesis.[21] It is generally accepted that TGF-β1 is locally produced in the peritoneum[3] and is likely to be the main growth factor for functional and structural changes of peritoneal membrane.[22,23] TGF-β1 gene transfer to rat peritoneum was shown to induce peritoneal fibrosis, neoangiogenesis, increase of peritoneal solute transport,[22] and epithelial–mesenchymal transition.[23] Our observations that omental and dialysate TGF-β1 increased in rats treated with PDS, that the expression of TGF-β1 coincided with functional and structural changes in peritoneal membrane, and that the increase in TGF-β1 was effectively prevented by NAC strongly suggest the involvement of ROS in peritoneal alterations.

VEGF is a mitogen for endothelial cells and a regulator of normal and abnormal angiogenesis[24] and induces vascular hyperpermeability by a direct action on endothelial cells.[25] VEGF is locally produced in the peritoneum,[3,26,27] has been identified in biopsy samples from long-term PD patients,[26] and dialysate VEGF was shown to correlate with solute transport.[27] eNOS also plays a role in the control of vascular tone and permeability[28,29] as well as angiogenesis via an interaction with VEGF.[30,31] Functional significance of eNOS in the peritoneal membrane permeability has been reported in acute peritonitis mouse model.[32] In this study, PDS-induced increase in omental and dialysate VEGF and peritoneal eNOS expression was significantly prevented by NAC, again suggesting the involvement of ROS in functional as well as structural changes in the peritoneum.

Ang II is a growth factor that regulates cell proliferation, apoptosis, and fibrosis.[33–35] Recently, we demonstrated that high glucose increases cellular and secreted Ang II in cultured HPMC, that Ang II generates cellular ROS, and that Ang II-induced upregulation of TGF-β1 and fibronectin was inhibited by catalase and diphenyleneiodinium, an nicotinamide

adenine dinucleotide phosphate oxidase inhibitor, in HPMC.[7] These observations suggested that the effect of Ang II on peritoneal changes is mediated by ROS. Our current observation that losartan effectively lowers omental LPO level and, at the same time, prevents peritoneal hyperpermeability, eNOS expression, and membrane thickening confirms that the effect of Ang II on peritoneal changes is mediated by ROS. The involvement of nicotinamide adenine dinucleotide phosphate oxidase in Ang II-induced ROS generation has also been identified in vascular smooth muscle cells[36] and endothelial cells.[37] A study by Hsieh *et al.*[38] showed that high glucose enhances angiotensinogen gene expression via ROS generation in rat kidney proximal tubular cells.

Interestingly, we found that NAC decreases dialysate Ang II. This suggests that ROS are not only downstream but also upstream signaling molecules to Ang II in PDS-induced peritoneal injury. We also observed that the increase in dialysate Ang II induced by PDS was prevented by losartan, suggesting a positive feedback system by which the internalized Ang II is responsible for enhanced generation of Ang II. Such a link between Ang II receptor activation and stimulation of local Ang II formation is in agreement with previous studies,[39–41] which showed that Ang II type 1 receptor stimulation augments intrarenal Ang II in Ang II-induced hypertension. The beneficial effect of losartan in the present study is consistent with an earlier study showing that intraperitoneal enalapril improves ultrafiltration capacity and decreases peritoneal thickening.[42]

GDP can induce intracellular ROS and signal through ROS[10,11] and are likely to contribute to conventional PDS-induced peritoneal alterations. We, however, did not use a control GDP-depleted PDS in this study because we found that GDP-depleted PDS generated almost as much ROS in HPMC as PDS containing high concentration of GDP (data not shown) probably from high glucose.

In conclusion, the present study strongly suggests that ROS generated by conventional PDS, regardless of whether produced by high glucose, Ang II, or GDP, may be responsible for progressive membrane hyperpermeability, neoangiogenesis, accumulation of extracellular matrix, and eventual peritoneal fibrosis. Thus, antioxidants or Ang II receptor blockers may allow better preservation of the structural and functional integrity of the peritoneal membrane during long-term PD.

MATERIALS AND METHODS

All chemicals and materials, unless otherwise stated, were obtained from Sigma Chemical Company (St Louis, MO, USA) and Becton Dickinson Labware (Lincoln Park, NJ, USA).

Animals and treatment

Male Sprague–Dawley rats weighing 250 g were purchased from Bio Genomics Inc. (Seoul, Korea) and housed in a room under controlled temperature ($23 \pm 1^{\circ}C$) and humidity (45–65%), 12-h light/dark cycle, and free access to water and chow (Samyang rat food, Seoul, Korea). All animal experiments were conducted in accordance with the National Institute of Health guide for the care and use of laboratory animals. Rats were randomized to four groups ($n = 8$ for each group): control, PDS, PDS with an antioxidant, and PDS with an Ang II receptor blocker. Commercial PDS containing 3.86% glucose, 20–30 ml depending upon the body weight change over time, with or without NAC 10 mM or losartan 5 mg/kg was administered intraperitoneally twice a day for 12 weeks. Dose of NAC was chosen based on the previous study[43] and our own preliminary data using 5, 10, and 50 mM and that of losartan based on the previous study.[44] pH of PDS was neutralized by NaOH and the final pH was 7.4. Control rats received sham injection (puncture of the peritoneal cavity without PDS) twice a day over 12 weeks. Peritoneal equilibration test was performed over 4 h before being killed. Rats were killed using pentobarbital sodium, blood and dialysate samples were collected, and omental tissue, liver, and anterior abdominal wall apart from injection site were removed and frozen until analysis. There was no evidence of infection at the puncture site or in the peritoneal cavity from repeated punctures. One animal each from control and losartan groups died during the study period.

Peritoneal equilibration test

Peritoneal equilibration test was performed using 30 ml of 3.86% glucose PDS. Four hours later, a tail blood sample and a dialysate sample were obtained. The dialysate to plasma creatinine ratio at 4 h (D_4/P_4 creatinine) was used as the solute transport parameter. The drained volume was calculated as the sum of peritoneal fluid aspirated and the weight of (wet-dry) gauze at 4 h. The creatinine concentration in the PD effluent was measured by modified Jaffe method and corrected for glucose interference by a correction factor derived by our laboratory.

Enzyme-linked immunosorbent assay

Total TGF-β1 (after acid activation) and VEGF were measured in dialysate and omental tissue using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) kit for TGF-β1 (R&D Systems, Minneapolis, MN, USA) and VEGF (R&D systems) according to the manufacturer's descriptions. Omental tissue was homogenized in a standard lysis buffer containing protease inhibitors (20 mM Tris-HCl, 1% Triton X-100, 137 mM sodium chloride, 5 mM ethylenediaminetetraaceticacid, 1 mM ethyleneglycol tetraacetate, 0.2 mM phenylmethylsulfonylfluoride, 10 μM leupeptin, 1 μg/ml aprotinin). Ang II was measured in dialysate using a commercial ELISA kit (Peninsula Laboratories, Belmont, CA, USA). Before the detection of Ang II, 1 ml of dialysate samples were lyophilized and reconstituted in 60 μl of H_2O.

Western blot analysis

Protein was extracted from frozen omental tissue by homogenization in a lysis buffer with the same composition as was used for ELISA. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane using a transblot chamber with Tris buffer (0.025 M Tris-HCl, 0.192 M glycine, and 20% MeOH). Equal amount of samples were verified by Ponceau S staining. The membrane was blocked for 1 h at room temperature with 5% non-fat milk in Tris-buffered saline-Tween 20. Membranes were incubated at 4°C overnight with rat monoclonal antibody to collagen I (Southern Biotech, Birmingham, AL) or hsp 47 (Stressgen, Victoria, BC, Canada). After extensive washing in Tris-buffered saline-Tween 20, the membranes then were incubated with horseradish peroxidase-conjugated anti-mouse immunoglobulin G or anti-goat immunoglobulin G for 1 h at room temperature. After washing, the membranes were incubated with enhanced chemiluminescence

system detection kit (Amersham Life Science, Little Chalfont, UK). Positive immunoreactive bands were quantified densitometrically.

Lipid peroxidation

The omental tissue samples were homogenized in a buffer solution containing 50 mM Tris-HCl (pH 7.4) and 1.15% KCl, and then centrifuged. The supernatant was used for the assay. The levels of lipid peroxidation were measured by thiobarbituric acid method with a modification as described previously.[45]

Histological assessment

Tissue samples obtained from the liver and anterior abdominal wall were fixed in a sufficient amount of 4% phosphate-buffered formaldehyde for 24 h, paraffin-processed and embedded, and 3 μm sections were cut. Cut sections were stained with Masson's trichrome stain. In histological assessment, attention was paid to the morphologic features of the mesothelial surface and submesothelial compact zone. The integrity of the mesothelial cell monolayer was assessed and the maximal thickness of the submesothelial compact zone was measured in subhepatic and parietal peritoneal membrane using Image Pro Plus (Media Cybernetics, Silver Spring, MD, USA).

Four-micrometer paraffin sections were processed for immunohistochemistry using avidin–biotin–horseradish peroxidase technique, as described previously.[46,47] The sections were deparaffined with xylene and ethanol, rinsed in tap water, and incubated with 3% H_2O_2 for 30 min to eliminate endogenous peroxidase activity. Before incubation with primary antibody, the sections were permeabilized by incubation for 15 min in 0.5% Triton X-100 in phosphate-buffered saline, blocked subsequently with blocking serum for 30 min, and incubated overnight at 4°C with the antibody against eNOS (1:1000; Transduction Lab., Lexington, KY, USA) diluted in phosphate-buffered saline. After washing in phosphate-buffered saline, the sections were incubated for 2 h with the biotinylated secondary antibody against mouse immunoglobulin G, and subsequently with the Vectastain ABC reagent (Vectastain ABC kit; Vector Laboratories, Burlington, CA, USA) for 2 h. After rinsing with phosphate-buffered saline, the sections were incubated with the peroxidase substrate solution, a mixture of 0.05% 3,3′-diaminobenzidine, and 0.01% H_2O_2 for 2 min at room temperature. After rinsing with Tris-HCl buffer, the sections were dehydrated with graded ethanol and xylene, mounted in Canada balsam, and examined by light microscope.

Statistical analyses

The mean values obtained from each group were compared by analysis of variance with subsequent Fisher's significant difference method. Non-parametric analyses were also used where appropriate. A P-value <0.05 was used as the criterion for a statistically significant difference.

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REFERENCES

1. Williams JD, Craig KJ, Topley N, *et al.*, Peritoneal Biopsy Study Group. Morphologic changes in the peritoneal membrane of patients with renal disease. *J Am Soc Nephrol* 2002; **13**: 470–479.
2. Davies SJ, Phillips L, Naish PF, Russell GI. Peritoneal glucose exposure and changes in membrane solute transport with time on peritoneal dialysis. *J Am Soc Nephrol* 2001; **12**: 1046–1051.
3. Ha H, Cha MK, Choi HN, Lee HB. Effects of peritoneal dialysis solutions on the secretion of growth factors and extracellular matrix proteins by human peritoneal mesothelial cells. *Periton Dial Int* 2002; **22**: 171–177.
4. Ha H, Yu MR, Lee HB. High glucose-induced PKC activation mediates TGF-beta 1 and fibronectin synthesis by peritoneal mesothelial cells. *Kidney Int* 2001; **59**: 463–470.
5. Ha H, Song JS, Yu MR, Lee HB. Oxidative stress in peritoneal dialysis. *Lect Notes ICB Semin* 2004; **65**: 32–39.
6. Lee HB, Yu MR, Song JS, Ha H. Reactive oxygen species amplify protein kinase C signaling in high glucose-induced fibronectin expression by peritoneal mesothelial cells. *Kidney Int* 2004; **65**: 1170–1179.
7. Noh H, Ha H, Yu MR *et al.* Angiotensin II mediates high glucose-induced TGF-β1 and fibronectin upregulation in HPMC through reactive oxygen species. *Periton Dial Int* 2005; **25**: 38–47.
8. Mortier S, Faict D, Schalkwijk CG *et al.* Long-term exposure to new peritoneal solutions: effects on the peritoneal membrane. *Kidney Int* 2004; **66**: 1257–1265.
9. Mortier S, Faict D, Lameire NH, De Vriese AS. Benefits of switching from a conventional to a low-GDP bicarbonate/lactate-buffered dialysis solution in a rat model. *Kidney Int* 2005; **67**: 1559–1565.
10. Che W, Asahi M, Takahashi M *et al.* Selective induction of heparin-binding epidermal growth factor-like growth factor by methyllglyoxal and 3-deoxyglucosone in rat aortic smooth muscle cells. *J Biol Chem* 1997; **272**: 18453–18459.
11. Greenwel P, Dominguez-Rosales JA, Mavi G *et al.* Hydrogen peroxide: a link between acetaldehyde-elicited alpha1(I) collagen gene up-regulation and oxidative stress in mouse hepatic stellate cells. *Hepatology* 2000; **31**: 109–116.
12. Yim MB, Yim HS, Lee C *et al.* Protein glycation: creation of catalytic sites for free radical generation. *Ann NY Acad Sci* 2001; **928**: 48–53.
13. Wautier MP, Chappey O, Corda S *et al.* Activation of NADPH oxidase by AGE links oxidant stress to altered gene expression via RAGE. *Am J Physiol* 2001; **280**: E685–E694.
14. Lal MA, Brismar H, Eklof AC, Aperia A. Role of oxidative stress in advanced glycation end product-induced mesangial cell activation. *Kidney Int* 2002; **61**: 2006–2014.
15. Nishigawa T, Edelstein D, Du XL *et al.* Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* 2000; **404**: 787–790.
16. Nakai A, Satoh M, Hirayoshi K, Nagata K. Involvement of the stress protein HSP 47 in procollagen processing in the endoplasmic reticulum. *J Cell Biol* 1992; **117**: 903–914.
17. Moriyama T, Kawada N, Ando A *et al.* Up-regulation of HSP 47 in the mouse kidneys with unilateral ureteral obstruction. *Kidney Int* 1998; **54**: 110–119.
18. Razzaque MS, Taguchi T. Collagen-binding heat shock protein (HSP) 47 expression in anti-thymocyte serum (ATS)-induced glomerulonephritis. *J Pathol* 1997; **183**: 24–29.
19. Razzaque MS, Shimokawa I, Nazneen A *et al.* Age-related nephropathy in the Fischer 344 rat is associated with overexpression of collagens and collagen-binding heat shock protein 47. *Cell Tissue Res* 1998; **294**: 471–578.
20. Mishima Y, Miyazaki M, Abe K *et al.* Enhanced expression of heat shock protein 47 in rat model of peritoneal fibrosis. *Periton Dial Int* 2003; **23**: 14–22.
21. Border WA, Noble NA. Transforming growth factor β in tissue fibrosis. *N Engl J Med* 1994; **331**: 1286–1292.
22. Margetts PJ, Kolb M, Galt T *et al.* Gene transfer of transforming growth factor-β1 to the rat peritoneum: effects on membrane function. *J Am Soc Nephrol* 2001; **12**: 2029–2039.
23. Margetts PJ, Bonniaud P, Liu L *et al.* Transient overexpression of TGF-β1 induces epithelial mesenchymal transition in the rodent peritoneum. *J Am Soc Nephrol* 2005; **16**: 425–436.
24. Ferrara N. Role of vascular endothelial growth factor in the regulation of angiogenesis. *Kidney Int* 1999; **56**: 794–814.
25. Hippenstiel S, Krüll M, Ikemann A *et al.* VEGF induces hyperpermeability by a direct action on endothelial cells. *Am J Physiol* 1998; **274**: L678–L684.
26. Combet S, Miyata T, Moulin P *et al.* Vascular proliferation and enhanced expression of endothelial nitric oxide synthase in human peritoneum exposed to long term peritoneal dialysis. *J Am Soc Nephrol* 2000; **11**: 717–728.
27. Zweers MM, Struijk DG, Smit W, Krediet RT. Vascular endothelial growth factor in peritoneal dialysis: a longitudinal follow-up. *J Lab Clin Med* 2001; **137**: 125–132.
28. Kubes P. Nitric oxide affects microvascular permeability in the intact and inflamed vasculature. *Microcirculation* 1995; **2**: 235–244.

29. Kone BC. Nitric oxide in renal health and disease. *Am J Kidney Dis* 1997; **30**: 311–333.
30. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. *J Clin Invest* 1997; **100**: 3131–3139.
31. Murohara T, Asahara T, Silver M *et al.* Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 1998; **101**: 2567–2578.
32. Ni J, Moulin P, Gianello P *et al.* Mice that lack endothelial nitric oxide synthase are protected against functional and structural modifications induced by acute peritonitis. *J Am Soc Nephrol* 2003; **14**: 3205–3216.
33. Ruiz-Ortega M, Lorenzo O, Suzuki Y *et al.* Proinflammatory actions of angiotensins. *Curr Opin Nephrol Hypertens* 2001; **10**: 321–329.
34. Wolf G, Neilson EG. Angiotensin II as a renal growth factor. *J Am Soc Nephrol* 1993; **3**: 1531–1540.
35. Mezzano SA, Ruiz-Ortega M, Egido J. Angiotensin II and renal fibrosis. *Hypertension* 2001; **38**: 635–638.
36. Griendling KK, Sorescu D, Ushio-Fukai M. NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 2000; **86**: 494–501.
37. Li JM, Shah AM. Mechanism of endothelial cell NADPH oxidase activation by angiotensin II. *J Biol Chem* 2003; **278**: 12094–12100.
38. Hsieh TJ, Zhang SL, Filep JG *et al.* High glucose stimulates angiotensinogen gene expression via reactive oxygen species in rat kidney proximal tubular cells. *Endocrinology* 2002; **143**: 2975–2985.
39. Zou LX, Imig ZD, Von Thun AM *et al.* Receptor-mediated intrarenal angiotensin II augmentation in angiotensin II-infused rats. *Hypertension* 1996; **28**: 669–677.
40. Nishiyama A, Seth DM, Naver LG. Angiotensin II type 1 receptor-mediated augmentation of renal interstitial fluid angiotensin II in angiotensin II-induced hypertension. *J Hypertens* 2003; **21**: 1897–1903.
41. Kobori H, Prieto-Carrasquero MC, Ozawa Y, Naver LG. AT1 receptor mediated augmentation of intrarenal angiotensinogen in angiotensin II-dependent hypertension. *Hypertension* 2004; **43**: 1126–1132.
42. Duman S, Wieczorowska-Tobis K, Styszynski A *et al.* Intraperitoneal enalapril ameliorates morphologic changes induced by hypertonic peritoneal dialysis solutions in rat peritoneum. *Adv Periton Dial* 2004; **20**: 31–36.
43. Pat B, Yang T, Kong C *et al.* Activation of ERK in renal fibrosis after unilateral obstruction: modulation by antioxidants. *Kidney Int* 2005; **67**: 931–943.
44. Ferrario CM, Jessup J, Chappell MC *et al.* Effect of angiotensin converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin converting enzyme 2. *Circulation* 2005; **111**: 2605–2610.
45. Ha H, Yu MR, Kim KH. Melatonin and taurine reduce early glomerulopathy in diabetic rats. *Free Radic Biol Med* 1999; **26**: 944–950.
46. Han KH, Jung JY, Cha JH *et al.* 1,25-Dihydroxyvitamin D3 stimulates osteopontin expression in rat kidney. *Nephron Physiol* 2003; **93**: 76–86.
47. Han KH, Lim JM, Kim WY *et al.* Expression of endothelial nitric oxide synthase in developing rat kidney. *Am J Physiol Renal Physiol* 2005; **288**: F694–F702.